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Original Article

PLAC4 mRNA SNP in non-invasive prenatal testing of Down syndrome

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Abstract: The purpose of this study was to explore the mRNA expression level of *PLAC4* in the maternal plasma and the clinical value of its single nucleotide polymorphism (SNP) rs8130833 in non-invasive prenatal testing (NIPT) of Down syndrome. 40 pregnant women were collected in Tianjin Medical University General Hospital from January 2014 to December 2015. Amniotic puncture karyotype analysis was adapted to determine whether the fetuses with Down syndrome (DS) or not. 20 fetuses were diagnosed with Down syndrome and recorded as the DS group, and 20 fetuses were normal and recorded as the control group. Quantitative reverse transcription-PCR (qRT-PCR) was used to detect the mRNA expression level of *PLAC4* both in the whole blood and plasma of pregnant women. A/G polymorphism of rs8130833 was analyzed by pyrosequencing method using the cell-free fetal RNA (cff RNA) in maternal circulation. The mRNA expression level of *PLAC4* in DS group was higher than the control group, but the difference was not statistically significant (P > 0.05). A/G polymorphism of rs8130833 was about 2:1 in DS group, and it was nearly 1:1 in control group. The *PLAC4* mRNA SNP (rs8130833) has a certain value of research and application in NIPT of DS.

Keywords: Placenta-specific 4 (PLAC4), single nucleotide polymorphism (SNP), non-invasive prenatal testing (NIPT), Down syndrome (DS), pyrosequencing

Introduction

Non-invasive prenatal testing (NIPT), or non-invasive prenatal testing (NIPT), is a prenatal diagnosis with none or insignificant level of invasiveness, and has substantial medical importance [1]. With the development of cell-free fetal DNA (cff DNA) in maternal circulation and the emergence of high-throughput sequencing, NIPT can detect fetal chromosomal abnormalities [2, 3]. Currently, NIPT has been studied, as a powerful tool, in the genetic diagnoses of many diseases, such as lethal skeletal dysplasia, congenital adrenal hyperplasia and methylmalonic academia [4-6].

Down syndrome (DS), also known as trisomy 21, is a genetic disorder caused by a third copy of chromosome 21 [7]. DS was first found and characterized by English physician John Langdon Down in 1862 [8]. Globally, DS occurs about 1 in per 1000 births and results in about 17,000 deaths [9, 10]. The most typical feature of DS is intellectual disability, and other characteristic physical symptoms include small stature and facial dimorphisms [9, 11, 12]. Besides, people with DS are at increased risk of cardiac defects and certain blood diseases, such as congenital heart defect, epilepsy, leukemia, thyroid diseases, and mental disorders [13]. Moreover, it was reported that congenital heart disease was found in 40% of infants with DS [14]. There is no effective treatment for DS so far, and guidelines recommend screening for DS to be offered to all pregnant women [15]. Moreover, NIPT has been widely used in the clinic to classify low- and high-risk pregnant women with DS fetuses. Chiu *et al.* [16] used massively parallel genomic sequencing to quantify maternal plasma DNA sequences for the noninvasive prenatal detection of fetal DS. *PLAC4* gene (placenta-specific 4), which is transcribed from chromosome 21 in placental cells, is a potential marker for the

NIPT of DS in the fetus [17]. Single-nucleotide polymorphism (SNP) arrays allow for detection of consanguinity and most cases of uniparental disomy and provide a higher sensitivity to detect low-level mosaic aneuploidies [18]. In this article, *PLAC4* and the SNP rs8130833 were detected of the cell-free fetal RNA (cff RNA) in maternal plasma, in order to preliminarily explore the clinical value of them in the NIPT of DS.

Materials and methods

Cases collection and grouping

40 pregnant women were collected in Tianjin Medical University General Hospital from January 2014 to December 2015. 20 cases were diagnosed with DS fetuses by amniotic puncture karyotype analysis, and recorded as the DS group. The other 20 normal cases were diagnosed by the same method and recorded as the control group. Pregnant women in the two groups were singleton pregnancy, no medical and surgical diseases, no smoking history, and all of them provided written informed consent for the collection of samples and subsequent analysis. Our research protocol was approved by the ethical committee (Tianjin Medical University General Hospital).

Sample processing and cff RNA isolation

10 ml peripheral venous blood sample was drawn from every pregnant woman, and collected into ethylene diamine tetraacetie acid (EDTA) containing tubes within 30 min. The blood samples were centrifuged at 3000 r/min for 10 minutes at 4°C. The supernatant was placed in the frozen storage tube with RNA inactivated enzyme, and stored at -80°C. The cff RNA from the whole blood and the plasma was respectively extracted with the QIAamp RNA Blood Mini Kit (QIAGEN, Germany).

Quantitative reverse transcription-PCR

The cff RNA was subjected to reverse transcription by Sensiscript RT Kit (QIAGEN, Germany). The final cDNA was obtained, and stored at -20°C. Afterwards, Quantitative real-time PCR was performed to determine the cff RNA level of *PLAC4* with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, America). The primers set of *PLAC4* were designed with PyroMark Assay Design Software 2.0, and the primer sequences were 5'-TGC AAC ACC ATT TGG GTT AAA TA-3', and 3'-CCA TGT TTA GGC CAG ATA TAT TCG-5'. They were synthesized by Beijing Genomics Institute (BGI, Shenzhen, China). Moreover, the reaction was set up in a reaction volume of 50 μL, including 0.2 μl Taq DNA polymerase (5 U/μl), 1 μl dNTP, 2 μl primer mix, 2 μl Template, 10 μl buffer (5×) and 34.8 μl H_2O. The reaction conditions: 95°C for 3 min and 40 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 1 min.

Pyrosequencing

The PCR products were immobilized on streptavidin-coated sepharose beads. 2 μl reaction binding bead, 40 μl *PLAC4* PCR products and 38 ul binding buffer were fully mixed for 10 min in the 96-well plates (15-25°C). Next, the mixture was removed by aspirating the beads with a vacuum pump, and the beads were treated for approximately 5 s with 75% ethanol, 5 s with sodium hydroxide, and 5 s with washing buffer. Then the vacuum pump was closed, and the plate was heated at 85°C for 2 min to make nucleic acid denaturation. The plate was then removed from the plate holder and the samples were allowed to cool to room temperature (15-25°C) for at least 5 min, which made primer and template annealing hybrid. Afterwards, the reagents, including enzyme and substrate mixtures, and nucleotides were added to the cartridge. The cartridge and the reaction plate were put on the pyrosequencing detector (PyroMark Q96, QIAGEN) to sequence. Finally, the A/G ratio of the SNP site (rs8130833) was analyzed with the AQ model of PyroMark Q96 software.

Statistical analysis

The SPSS 22.0 statistical software (Chicago, IL, USA) was applied for statistical analysis. All experiments were performed in triplicate, and data were expressed as the means ± standard. For comparison between parameters of 2 groups, Fisher's F-test and student's t-test were used. $P < 0.05$ was considered as a significant difference.

Results

The mRNA expression level of PLAC4

Results of qRT-PCR in the whole blood and the plasma were separately showed in **Tables 1**

Table 1. The mRNA expression level of *PLAC4* in the maternal whole blood

Group	Case	PLAC4 mRNA	F value	*P* value
DS	5	184.85±45.84	0.62	0.45
control	5	182.69±37.65		

Table 2. The mRNA expression level of *PLAC4* in the maternal plasma

Group	Case	PLAC4 mRNA	F value	*P* value
DS	20	7.54±1.32	1.29	0.26
control	20	2.27±1.06		

and **2**. The mRNA expression level of *PLAC4* in DS group (184.85±45.84) was slightly higher than the control group (182.69±37.65) in the whole blood. Similarly, the mRNA expression level of *PLAC4* in DS group (7.54±1.32) was higher than the control group (2.27±1.06) in maternal plasma, but it was still no significant difference ($P > 0.05$).

A/G polymorphism of PLAC4 mRNA SNP

A/G polymorphism of PLAC4 mRNA SNP rs-8130833 was detected using pyrosequencing method. A/G polymorphism of rs8130833 was about 2:1 in DS group (**Figure 1A**), while it was approximately 1:1 in control group (**Figure 1B**). Besides, the site was found to be homozygote in 5 cases, containing 2 DS cases and 3 control cases, and the A/G ratios of them were nearly 1:0 (**Figure 1C**).

Discussion

Currently, DS was no cure, and screening might be the most effective preventive measures. The methods of prenatal diagnosis included measuring nuchal translucency in addition to blood tests for free or total beta human chorionic gonadotrophin (β-hCG) and pregnancy-associated plasma protein A (PAPP-A), detecting the maternal serum alpha-fetoprotein, unconjugated estriol, hCG, and inhibin-A, and DNA analysis of cell-free fetal DNA from the mother [15, 19]. The different screening techniques with varying levels of accuracy were from 67% to 99.9% [20-22]. All of them had some limits, and they might cause pregnancy loss in the non-sick fetus or other related complications. Moreover, some tests could be carried out only in the mid trimester of pregnancy. At that time, if the fetus was confirmed with DS, induced labor might be the only method to terminate the pregnancy. Besides, amniocentesis or chorionic villous sampling was still the "gold standard" to confirm the diagnosis, which was easy to cause infection [15]. Therefore, it was necessary to establish a non-invasive, rapid and effective prenatal diagnosis of fetal chromosomal diseases in the early pregnancy.

NIPT avoided or reduced the need for invasive diagnostic procedures, which was safer and more effective [1]. Besides, advantages of NIPT included the rapid turnaround, relatively low cost, and simplicity of the procedure for pregnant women [23]. Hence, NIPT might be a highly promising solution for above problems. However, current NIPT mainly detected the fetal DNA from the maternal cff DNA, which the accuracy rate was low and easy to be affected by gender. Detection of cff RNA in maternal circulation did not depend on sex of the fetus, and had the incomparable advantage. *PLAC4* mRNA, a chromosome-21 transcript, was a virtual preference for fetal-specific makers, which rapidly disappeared from maternal circulation after delivery and cannot be detected in the plasma of nonpregnant women [17]. In 2000, Poo *et al.* [24] found that the fetal specific mRNA encoded by placenta-derived *PLAC4* emerged in maternal plasma, which provided a possibility for the application of the free fetal mRNA in noninvasive prenatal DS screening. Furthermore, SNP was a kind of widely gene mutation in human genome with highly genetic stability and absolutely quantitative advantage [25]. It had been widely used in the detection of genetic diseases, such as the construction of genetic map, the evaluation of biological diversity, etc. Tsui *et al.* [26] reported that the quantification of the total *PLAC4* mRNA concentration could be used in a synergistic manner with the RNA-SNP allelic ratio approach to diagnose DS. Moreover, one study indicated that the sensitivity and specificity of mRNA-SNP *PLAC4* detection were 100% and 89.7% respectively in the DS diagnosis [26].

In this article, the *PLAC4* mRNA level of DS group was slightly higher than that of control group, but it had no significant difference in maternal plasma (**Table 2**). It was suggested that *PLAC4* mRNA from fetus was very little in maternal plasma. Furthermore, *PLAC4* mRNA

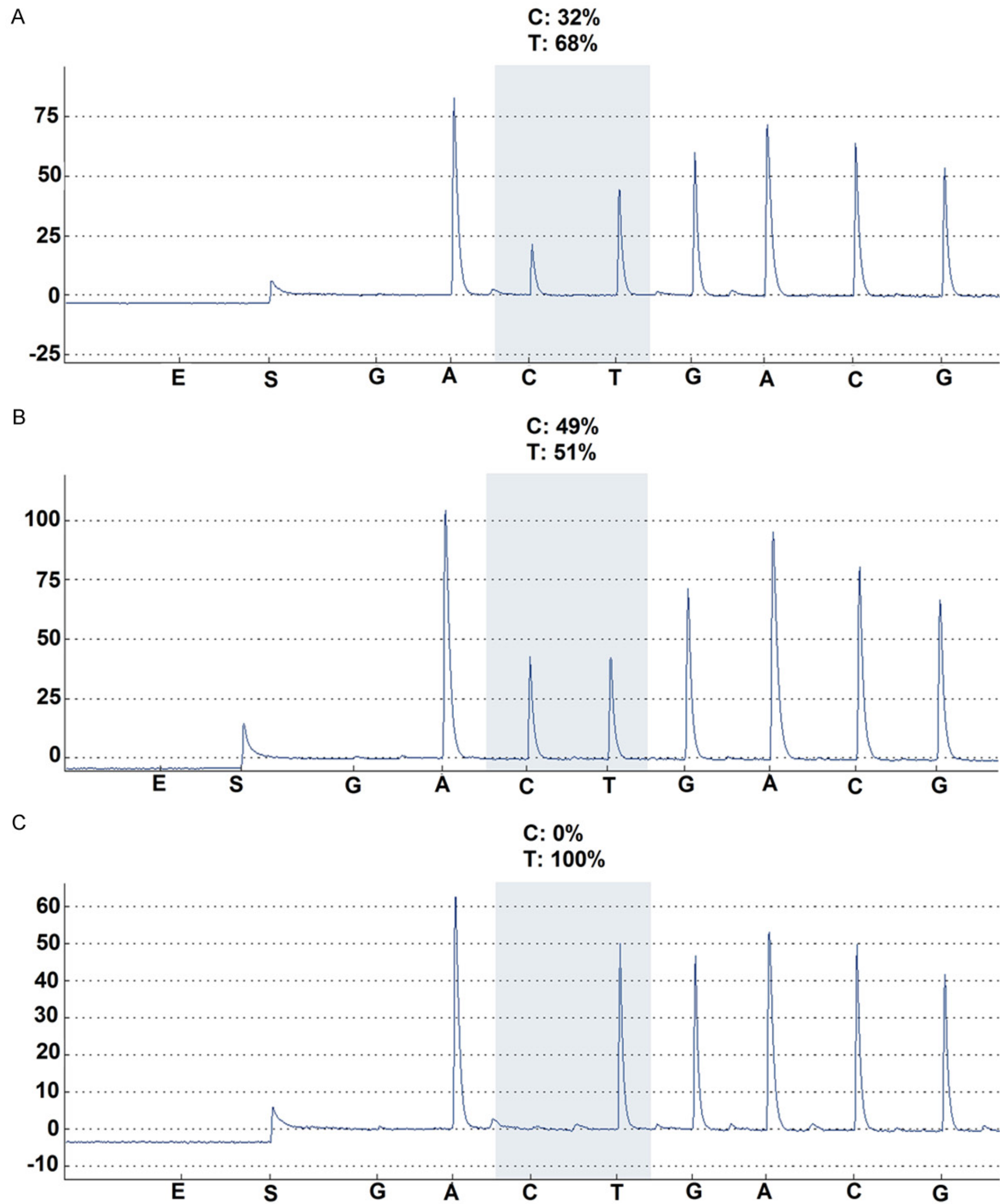

Figure 1. A/G polymorphism of PLAC4 mRNA SNP rs8130833.

from fetus in maternal plasma was much higher than that in the whole blood of pregnant woman. YMD Lo [27] showed that a heterozygous euploid fetus should yield equal proportions of each allele, giving an allelic ratio of 1:1, and a heterozygous triploid fetus would yield the allelic ratio 1:2 or 2:1. Results of A/G polymorphism of *PLAC4* mRNA SNP (rs8130833) showed that a heterozygous of DS cases was with a ratio 2:1 and a heterozygous of normal cases with a ratio 1:1. Therefore, the *PLAC4* mRNA SNP (rs8130833) might be used in NIPT of DS. Besides, this study had some limits. It was a small sample study, and only the hetero-

zygous fetus could be used to detect DS with the mRNA-SNP method. In the follow-up study, we would increase samples, combined detecting multiple SNP sites, to improve the coverage rate. Further exploration of the research and application values would be performed in the DS NIPT with the mRNA-SNP method.

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Disclosure of conflict of interest

None.

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